REPORT DOCUMENTATION PAGE

Form Approved
OBM No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	1997	Book

4. TITLE AND SUBTITLE
General Techniques for SEM Observation

5. FUNDING NUMBERS
Job Order No. 573505206
Program Element No. 0601153N
Project No.
Task No.
Accession No.

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Naval Research Laboratory
Oceanography Division
Stennis Space Center, MS 39529-5004

8. PERFORMING ORGANIZATION REPORT NUMBER
NRL/BA/7333--95-0006

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of Naval Research
800 North Quincy Street
Arlington, VA 22217-5000

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Procedures in Electron Microscopy, Chapter 14
John Wiley & Sons

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release. Distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

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14. SUBJECT TERMS	15. NUMBER OF PAGES
ESEM (environmental scanning electron microscope), microbiology, biofilms, cathodoluminescence imaging, high-pressure SEM, bacteria, algae, fungus, and polymers	25
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	SAR

Procedures in Electron Microscopy

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Block B, Union Industrial Building, Singapore 2057

British Library Cataloguing in Publication Data

A catalogue record for this book is available from the British Library

ISBN 0 471 92853 4

Typeset in 11/13pt Times by Page Bros (Norwich) Ltd.
Printed and bound in Great Britain by Orbital Press, Letchworth.

14 General Techniques for SEM Observation

* Not yet published.

Background Information

This minimization procedure is only necessary for samples that must be viewed in the hydrated state without prior drying. A more direct installation sequence may be used for initially dry samples.

For some samples, it may be important to avoid dehydration, but the presence of additional water is not a problem. In such cases it is recommended that the sample be covered in excess water before using the optimized installation sequence.

Troubleshooting

The response of the Peltier stage may not be sufficiently fast to keep the sample at a constant 3 °C during the optimized installation sequence, because of the heat changes due to evaporation and condensation. This is a limitation of the equipment and is difficult to correct. The more efficient the control system of the Peltier stage is, the better the optimization will be.

☐ Procedure

Examination of hydrated, nonconductive biofilms in ESEM

Characklis & Marshall (1990) define biofilms in the following terms:

- They are composed of microbial cells (principally bacteria, algae and fungi) immobilized at a substratum
- The cells of a biofilm may or may not be embedded in an organic polymer matrix of microbial origin
- They are surface accumulations that are not necessarily uniform in time or space
- They may contain a significant fraction of inorganic or abiotic substances held together by a matrix of biotic origin

These definitions are sufficiently broad to include a range of situations, from microorganisms growing on moist, subaerial surfaces to fully developed slime layers on submerged surfaces. Biofilms are a difficult class of specimens to examine with conventional scanning electron microscopy (SEM) because they are highly hydrated (≥ 95% water) and nonconductive.

Desiccation procedures necessary for conventional SEM cause collapse or dissolution of biofilm constituents. Artifacts introduced by a typical dehydration protocol (Little *et al.*, 1991) are illustrated in Plates 14:10.6a and b. Certain HPSEM technologies, such as the ElectroScan™ ESEM, avoid desiccation artifacts by using a water vapor atmosphere in the specimen chamber to produce a signal. Not only can the sample be maintained at virtually any degree of water saturation, but local electron charge build-up is dissipated by the water vapor, and the need for a heavy-metal coating that interferes with energy dispersive X-ray spectroscopy (EDXS) is eliminated. In most cases, specimens may be viewed in ESEM with little preparation.

Equipment

- ☐ ESEM
- ☐ Peltier cooling stage
- ☐ Specimen stub for cooling stage (concave on one end)
- ☐ Containers, tubes or plates (to hold specimen for rinsing)
- ☐ Pipettes
- ☐ Tweezers

Reagents

- T ■ 2–4% glutaraldehyde in buffer (0.1 M cacodylic acid or natural or artificial seawater at ambient salinity (as needed)
- T ■ 5% solution of osmium or ruthenium tetroxide (as needed)
- ☐ Distilled water
- ☐ Salinity series, from ambient salinity to distilled water (as needed)

T toxic

Method

The basic procedure assumes that the specimen has excessive salts or nutrients, has been fixed with buffered glutaraldehyde and is small enough to fit in the concavity of the Peltier cooling stage stub. Other situations will be discussed below.

1. Rinse salts, fixatives or excess nutrients from the specimen by immersing or covering it with distilled water for several minutes. If the specimen is from marine waters, pass it through a series of decreasing salinity rinses, e.g. 100% seawater, 75% seawater:25% distilled water, 50:50, 75:25 and, finally, 100% distilled water. Use chilled solutions.
2. Pre-chill the Peltier cooling stage in a freezer for 30 min. Chill the specimen in a refrigerator.

3. Transfer the Peltier stage to the chamber. Read the temperature of the stage on the temperature controller (the temperature will have risen from 0 °C to about 2–3 °C), and set the controller to this temperature or slightly lower.

4. Load the specimen into the concavity of the stub and cover with distilled water.

5. Close the chamber door. Set the desired end pressure to ~5 Torr (~665 Pa) using the vapor pressure gauge and the 'set' button to initiate pumpdown.

6. When the pre-set pressure is reached, the isolation valve will automatically open and an image will be displayed on the monitor. The overlying layer of water will be visible.

7. Use the manual pressure fine control to slowly decrease pressure (0.5 Torr or ~65 Pa increments) to remove the overlying water (too rapid a pumpdown when the sample is at 0–3 °C may freeze the specimen by evaporative cooling). Monitor the live image closely during pumpdown. The specimen will slowly emerge without distortion until it is completely uncovered (see, for example, Plates 14:10.7a–c).

8. Once the desired amount of overlying water has been removed from the specimen, press the 'set' button to automatically maintain the chamber pressure. Adjust the chamber pressure using the pressure fine control only to change the hydration of the specimen. If examination must be interrupted, the specimen can easily be flooded again to prevent dehydration by injecting water vapor into the chamber using the fine control or the 'flood' button.

9. Operating parameters—use nominal or default microscope operating parameters: an accelerating voltage of 20 keV, default spot size, beam current at filament saturation, a detector distance of ~14 mm and a scan rate of ~2 frames per second. The final chamber pressure for adequate image resolution will be ~4 Torr (~530 Pa) at a specimen temperature of ~3 °C.

10. Bring the specimen stage up to ~7–8 mm from the ESD detector to increase the resolution, and adjust the brightness and contrast as necessary to give a good image.

11. Use the digital image 'integrate' or 'add' function to increase the resolution or to record the image rather than slowing the scan rate.

Applications

The following list with examples gives some idea of the range of specimens to which this technique can be applied:

- Algal biofilms on stone (Plate 14:10.7c)
- Biofilms on painted surfaces (Plate 14:10.8)
- Biofilms on metal surfaces, e.g. cooling/heat exchanger tubing (Plates 14:10.6 and 14:10.9)
- Suspended marine aggregates ('marine snow') (Plate 14:10.10)
- Mixed biofilms on organic synthetics (Plate 14:10.11)
- Algal biofilms on vascular plants (Plate 14:10.12)
- Fungi on agar (Plate 14:10.13)
- Internal organelles and parasites (Plate 14:10.14)

Commentary

The basic considerations for viewing hydrated, nonconductive specimens in ESEM are as follows:

(a) Sample preparation.

(b) Control of specimen temperature.

(c) Microscope operating parameters.

Specimens requiring no pretreatment

Subaerial biofilms and biofilms that developed in a freshwater environment may be inserted directly into the ESEM chamber without fixation or other preparation. As noted below, some distortion may occur due to localized heating and dehydration.

Fixed versus unfixed specimens

Unfixed specimens containing microorganisms that have a rigid structure, such as diatoms and many bacteria, may be viewed in ESEM with no damage, but microorganisms having less rigid cell walls, such as fungi, may show some collapse (Plate 14:10.13). If the analysis is compromised by this effect, a lower beam current or accelerating voltage must be used that generates less localized heating, or the sample must be fixed to toughen cell walls.

If preservation of cellular and matrix morphology is important, the sample must be fixed. Glutaraldehyde is the fixative of choice (see Module 5:1). Glutaraldehyde kills microorganisms, and it cross-links organic polymers, both in the biofilm matrix and cell walls, thereby increasing the mechanical strength of these structures without the shrinkage associated with formaldehyde. Electron microscopy grade glutaraldehyde is recommended to minimize the presence of polymerized glutaraldehyde (see Module 5:1). Polymerization is encouraged by heat, so glutaraldehyde and samples immersed in glutaraldehyde solution should be stored under refrigeration.

Glutaraldehyde solution is slightly acidic and should be buffered with 0.1 M sodium cacodylate. For marine biofilms 0.22 mm-filtered natural or artificial seawater may be used. The carbonate system naturally present in seawater provides the buffering action.

! Further fixation of the sample may be provided by exposing it to osmium tetroxide solution or vapor (see Module 5:1). An alternative fixative is ruthenium tetroxide, the EDXS peak of which interferes only with chlorine, as opposed to osmium, the EDXS peaks of which interfere with silicon and potassium (Lavoie, 1992). These heavy metal oxides are even more toxic than glutaraldehyde, and they are strong oxidizers of organics such as plastics, paper and skin. They should always be ordered with a material safety data sheet and be handled carefully.

Rinsing

If the specimen has been fixed, or is from a marine or brackish environment, or has a high concentration of nutrients and salts, it must be rinsed before being inserted into the microscope. Salts will crystallize as water is withdrawn from the sample, and glutaraldehyde (and cacodylate) will slowly polymerize under the electron beam to form a continuous, obscuring coating over the specimen. This latter effect is shown in Plate 14:10.15. Osmotic shock from rinsing specimens from concentrated ionic media directly in distilled water does not seem to be a problem, but standard protocol calls for putting this type of specimen through a series of decreasingly concentrated solutions.

Microorganisms associated with biofilms are generally adapted to cope with low or changing osmolarity in their immediate environment. Build-up of internal pressure due to inward diffusion of water that could lead to cell lysis is prevented by rigid cell walls. Bacteria typically found in biofilms may have internal osmotic pressures as high as 10–25 atmospheres (Characklis *et al.*, 1990). The fact that ionic concentration (and thus osmolarity) can vary radically throughout typical biofilms indicates that the biofilm constituents (exopolymers and microbes) would not be present if they could not withstand osmotic extremes. Osmotic damage to microorganisms due to rinsing with distilled water should be visible as burst cells, although this condition has never been observed, even in dealing with biofilms generated in marine environments. Likewise, biopolymers, when present, are heterogeneous and cross-linked, creating a robust structure (Christensen & Characklis, 1990). Biofilms are variable, however, and the prudent researcher will experiment with fixation and rinsing procedures to produce optimum results.

Pumpdown and specimen hydration

Biofilm specimens from aqueous environments can be conveniently immersed in distilled water in the concave end of the specimen stub. Larger, flat specimens may be placed on the flat end of the inverted stub for more contact area and covered with distilled water. If the specimen is such that it does not permit standing water, the operator will have to work as quickly as possible to avoid dehydration of the specimen. Dehydration is minimized in any case, because the specimen chamber is automatically maintained at water vapor saturation during pumpdown from atmospheric pressure to working pressure. Once the working pressure is fixed using the 'set' button, the instrument periodically injects water vapor into the chamber to automatically maintain that pressure. During the final stage of the removal of overlying water, the live image is the best gauge of specimen hydration during pumpdown and should be closely monitored.

Specimen cooling, localized heating

Sample temperature is critical to sample hydration and is modulated by an electronically cooled Peltier stage. This device has a limited cooling rate and capacity. Small specimens that fit into the concave stub can be temperature-controlled for extended periods. The ability of the Peltier stage to keep a larger specimen cooled depends on the specimen size, thermal conductivity and initial temperature. Specimen size and thermal conductivity determine the rate at which heat produced by the electron beam arrives at the stage for removal. Thermal conductivity and initial temperature determine how long the specimen can be viewed before localized heating becomes a problem. Localized heating will be exacerbated if the specimen substratum is not thermally conductive. In addition, some substrata, such as ceramics, efficiently convert electron beam energy to heat as well, possibly overwhelming the capacity of the Peltier stage to extract heat.

The operator can monitor localized heating only visually. The chamber pressure and stage temperature are only proximal readings. Especially in larger specimens, the temperature of the irradiated portion of the specimen will always be somewhat above the stage temperature reading. In severe situations, even fixed specimens will exhibit distortion from dehydration or boiling due to localized heating. Pre-chilling will delay localized heating and will permit better control of specimen saturation through chamber pressure manipulation. The beam current, the accelerating voltage, the spot size, the scan rate, the magnification and time under the beam all have an effect on localized heating.

Finally, in some models of the ElectroScan™ ESEM, a lighted viewing port is mounted in the chamber for visually monitoring samples (the environmental secondary detector—ESD—is not sensitive to light). The viewing light should be used sparingly, as it introduces noticeable heat into the chamber.

Microscope operating parameters

Operating parameters should be chosen to use the minimum energy necessary to obtain a usable image. Any increase of electron energy into the sample may exacerbate localized heating. To begin, the spot size and beam (emission) current should be set to nominal or default values. The spot size may be decreased to improve resolution or penetration to image internal features. Deeper penetration may also be accomplished by increasing the accelerating voltage and/or beam current (by means of the bias control) over that required for saturation. All three actions increase energy input to the specimen.

A slow scan rate may improve resolution (especially at low signal strengths), but will also put more energy into the sample per unit time. A scan rate of ~2 frames per second is adequate for surveying a specimen. For better resolution of a particular field when viewing or recording, the digital 'integrate' or 'add' function should be used rather than slowing the scan rate. Note that the standard function for recording an image on photographic film involves a slow scan rate, and, in susceptible specimens, thermally induced movement during recording may result in smeared lines in the micrograph. To photograph the digital image, one must expose the film to the digital image more than once to build up the image on the film or increase the recording CRT brightness to capture the image in one exposure.

The distance between the detector and the sample should be maximized during pumpdowns to prevent liquid water from being drawn up into the column (there is a constant flow of atmosphere from the chamber into the differentially pumped column). After the specimen is exposed, the working distance is reduced to decrease scattering of the electron beam by water molecules and thereby increase signal strength and resolution.

Aside from the operating parameters mentioned above, sample chamber pressure is the most important parameter under operator control. The environmental secondary detector (ESD) in ESEM is also a pressure-limiting aperture between the specimen chamber and the first portion of the differentially pumped electron column. The larger the detector aperture is, the lower the possible magnification. A detector with a 1000 μm aperture allows magnifications as low as 250× with specimen chamber pressures theoretically as high as 10 Torr (1330 Pa); however, excessive scattering of the electron beam by water molecules at pressures this high yields low signal strength, so lower pressure is recommended for better resolution and a reasonably fast scan rate. Theoretical temperature and pressure combinations that yield saturation for water vapor are given in Table 14:10.1.

Table 14:10.1 Temperature versus pressure to maintain a saturated water vapor atmosphere (after Weast & Astle, 1980)

Temperature (°C)	Vapor pressure (Pa)	Vapor pressure (Torr)
9	1145	8.6
8	1071	8.0
7	999	7.5
6	932	7.0
5	870	6.5
4	811	6.1
3	757	5.7
2	704	5.3
1	656	4.9
0	609	4.6
−1	563	4.2
−2	519	3.9
−3	465	3.5

In practice, for a given Peltier stage temperature, the chamber pressure will have to be lower than that for water vapor saturation to expose the specimen for observation. Again, the best gauge is visual monitoring of the sample during pumpdown. The parameters given above provide a good balance between resolution, scan rate and sample hydration.

EDXS Analysis

EDXS must be performed at lower pressures than those used for viewing, especially if element X-ray maps are desired. Although the ESD detector requires water vapor to generate an image signal, X-rays emitted by the specimen are absorbed by water molecules. In addition, depending on the chamber geometry, the sample may require tilting from horizontal (the viewing angle) toward the X-ray detector to increase the acceptance angle. This requires the stage to be moved away from the ESD detector, increasing the beam path length and beam scattering, and resulting in decreased X-ray production and spatial resolution (Griffin *et al.*, 1994). The accelerating voltage may be increased to compensate. For all these reasons, chamber pressures of ≤ 2 Torr (≤ 265 Pa) are necessary for adequate X-ray count rates and spatial resolution (Griffin *et al.*, 1994); unfortunately, some specimens may exhibit signs of dehydration at such a low pressure. In practical terms, these considerations mean that observations should be completed first and X-ray analyses second.

Viability, Sample Desiccation and Viewing Time

Short viewing times in ESEM may not necessarily kill microorganisms within biofilms. Fungi and bacteria may remain viable and be re-examined with multiple viewings for time-series studies (Plate 14:10.13).

Desiccation, either during observation in the ESEM or during storage, may not do irretrievable damage to many types of biofilms. Some shriveling of biopolymer matrix may be observed, but with some specimens this may be totally reversed upon rehydration. Lavoie *et al.* (1995) reported marine snow samples that were observed through several desiccation–rehydration cycles. Glutaraldehyde-fixed, dehydrated biofilms have been stored and rehydrated for viewing several times with no gross differences noted in bacterial or biopolymer distribution and morphology.

The time for which a specimen can be viewed in ESEM is largely determined by its heat transfer characteristics. Typically, biofilms on metal substrata can be viewed without visible damage for as long as 45 min using the operating conditions stated. Lingering in one spot for long periods or at high magnifications exacerbates localized heating.

Expected Results

The answer to the most important question, 'What can we expect to see?', depends largely upon the nature of the biofilm. If the biopolymer is discontinuous or very thin, as in Plates 14:10.7c, 14:10.8, 14:10.9 and 14:10.10, most particulate constituents will be visible along with any tenuous polymer strands. Thick or continuous polymers, however, will show little of the particulate constituents, as in Plate 14:10.11, because, like conventional SEM technology, ESEM generally is limited to imaging the specimen surface. Some specimens may appear to be partially transparent (Plates 14:10.8 and 14:10.9), and, in particular, some internal features may be revealed (Plate 14:10.14). It also may be possible to view internal structures by using heavy metal stains and the backscatter signal. Collins *et al.* (1993) reported successful viewing of internal cellular organelles in this manner.

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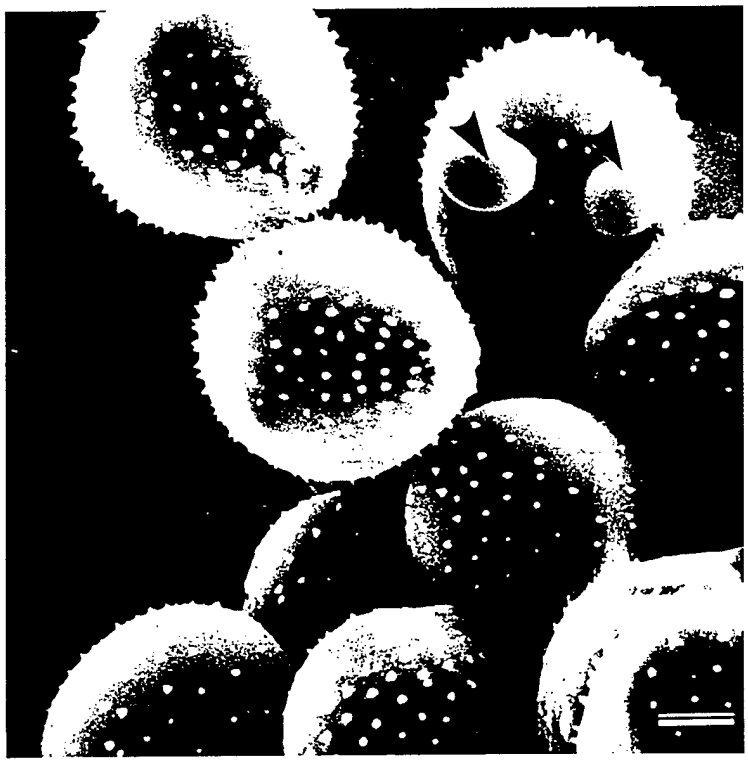

Plate 14:10.3 Secretion of fluid (arrows) from uredospores of the bean rust fungus *Uromyces viciae-fabae* (MEATSEM). Bar = 10 μm.

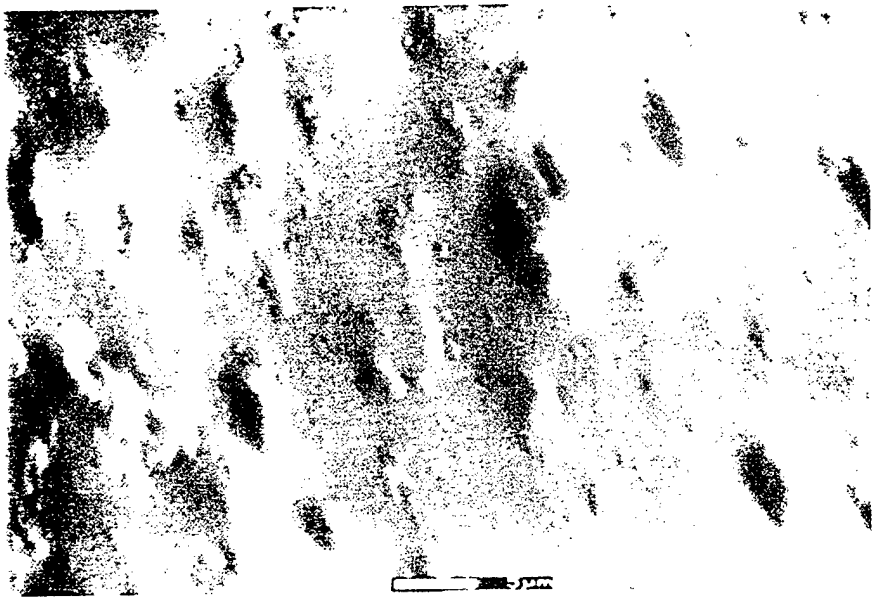

Plate 14:10.4 A micrograph of double cream taken 10 minutes after completing the installation sequence described. Bar = 5 μm. From Cameron & Donald (1994); reproduced by permission of *The Royal Microscopical Society*.

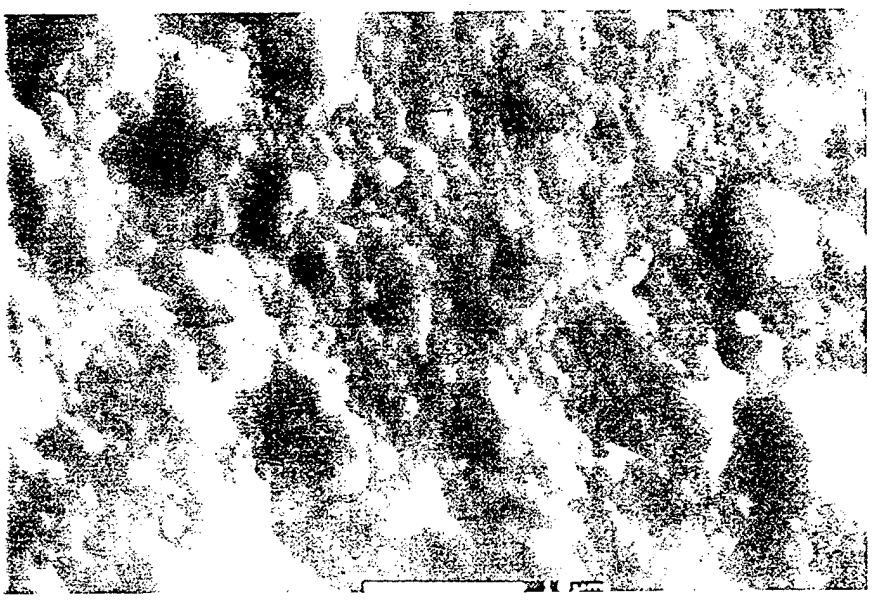

Plate 14:10.5 A micrograph of double cream taken 10 minutes after completing a nonoptimized installation sequence suggested in the manual. Bar = 10 μm. From Cameron & Donald (1994); reproduced by permission of *The Royal Microscopical Society*.

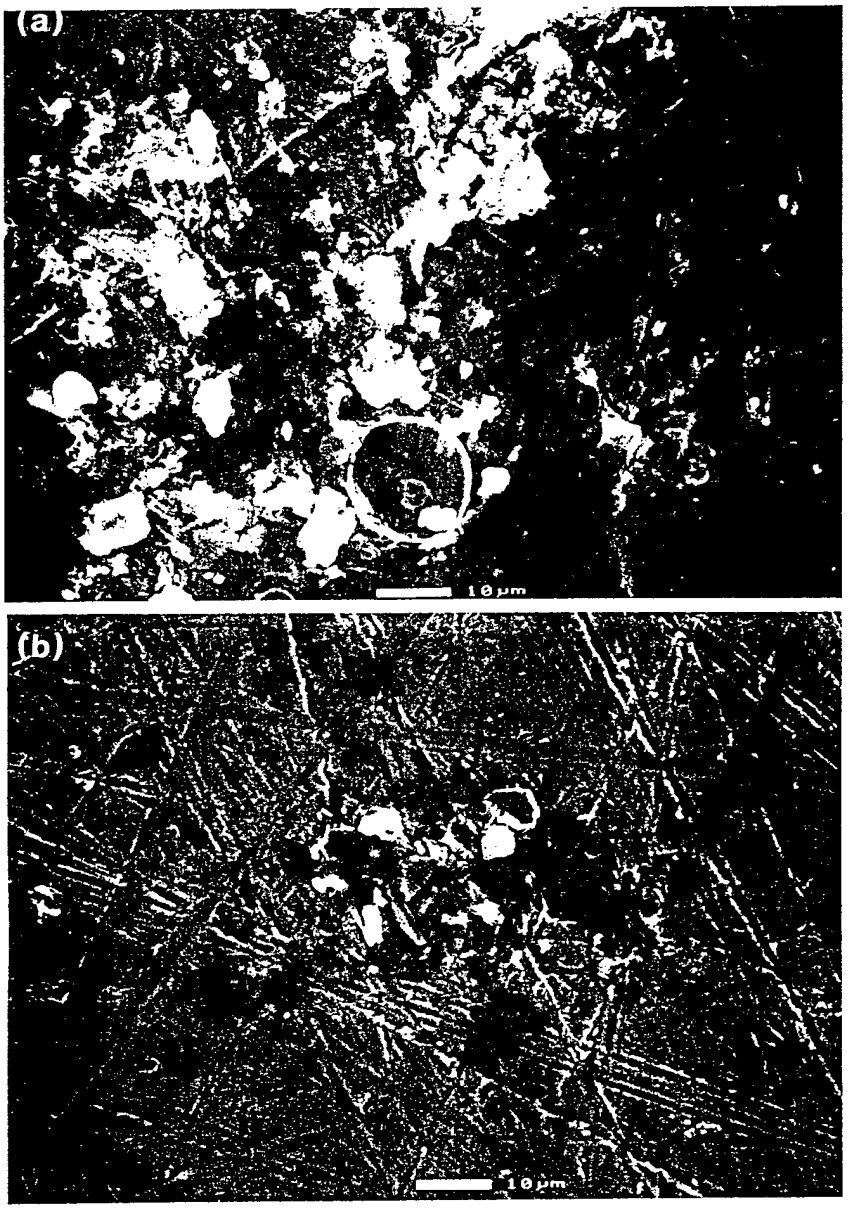

Plate 14:10.6 The effect of the conventional chemical dehydration procedure. (a) An ESEM image of wet estuarine biofilm on stainless steel substratum: (b) an ESEM image of the same sample after standard treatment with acetone and xylene. Bars = 10 µm.

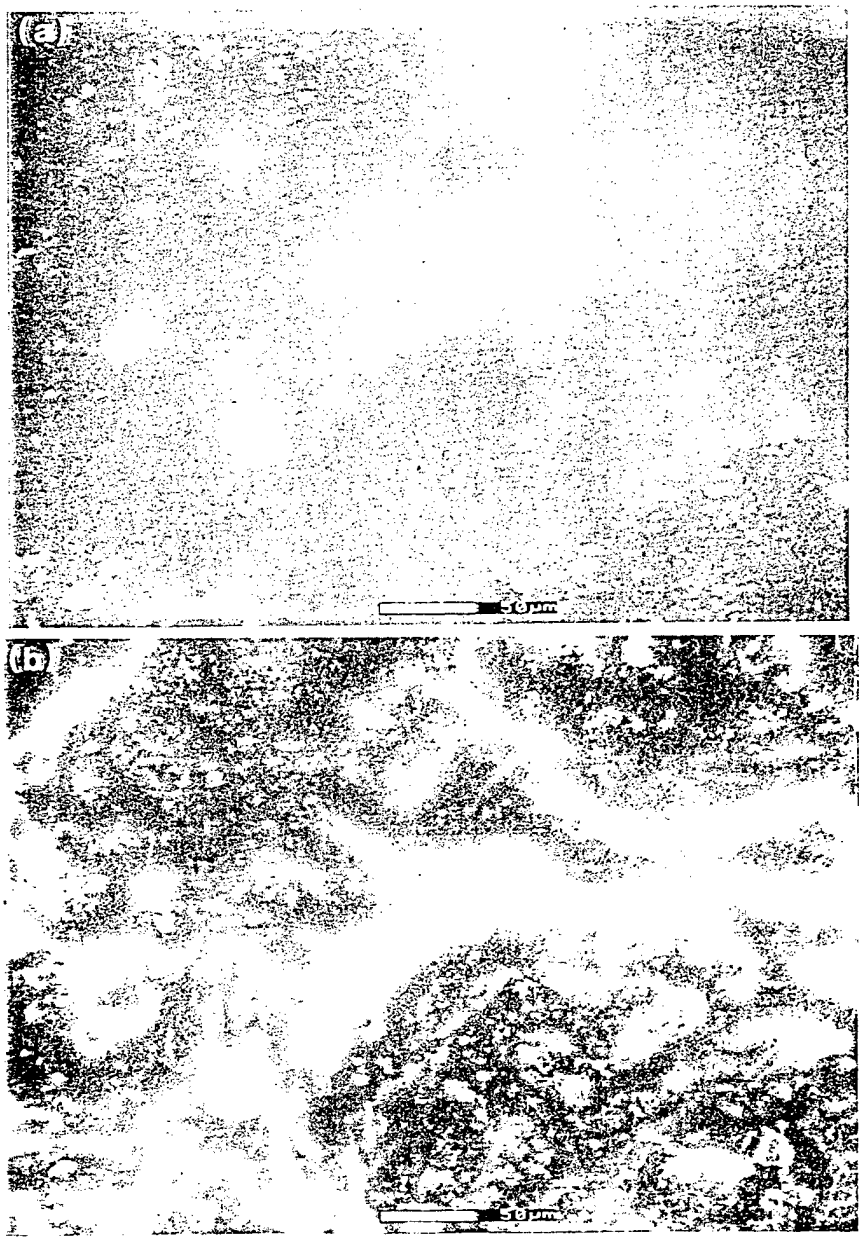

Plate 14:10.7 Typical images during a pumpdown sequence, natural biofilm on stone, unfixed sample. (a) Features just visible beneath overlying water, 6.2 Torr (824 Pa); (b) salient features visible, with dark areas of liquid water in depressions, 4.5 Torr (603 Pa); (c) features fully exposed in the stable hydration state, 4.1 Torr (550 Pa). Bars = 50 μm.

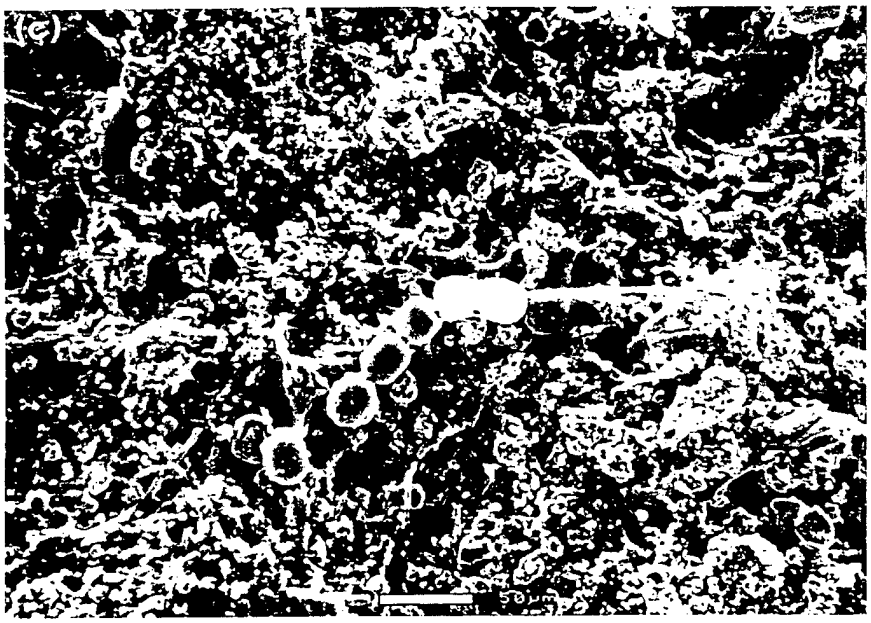

Plate 14:10.7—*continued.*

Plate 14:10.8 A laboratory-grown biofilm on painted metal, consisting mostly of bacteria, grown in nutrient enriched medium and fixed with buffered glutaraldehyde. Note that the cells tend to be semitransparent in ESEM under these conditions. Bar = 5 μm.

Plate 14:10.9 A laboratory-grown biofilm consisting of bacteria of the genus *Oceanospirillum* and polymer matrix growing on copper in unenriched artificial seawater, fixed with buffered glutaraldehyde. Bar = 2 μm.

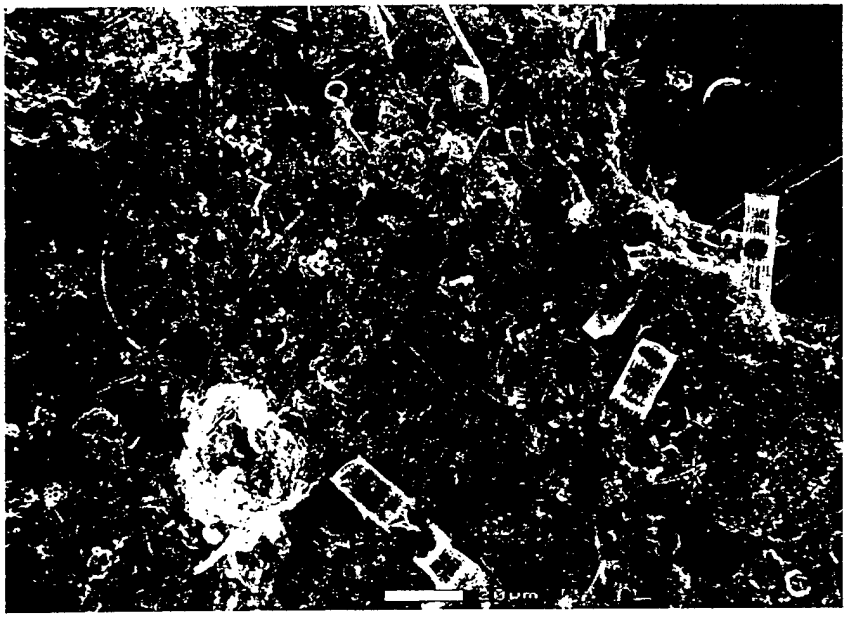

Plate 14:10.10 'Marine snow', i.e. marine suspended particulate. A very thin polymer matrix connects a heterogeneous collection of inorganic and biological particles: fixed with buffered glutaraldehyde. Bar = 20 µm.

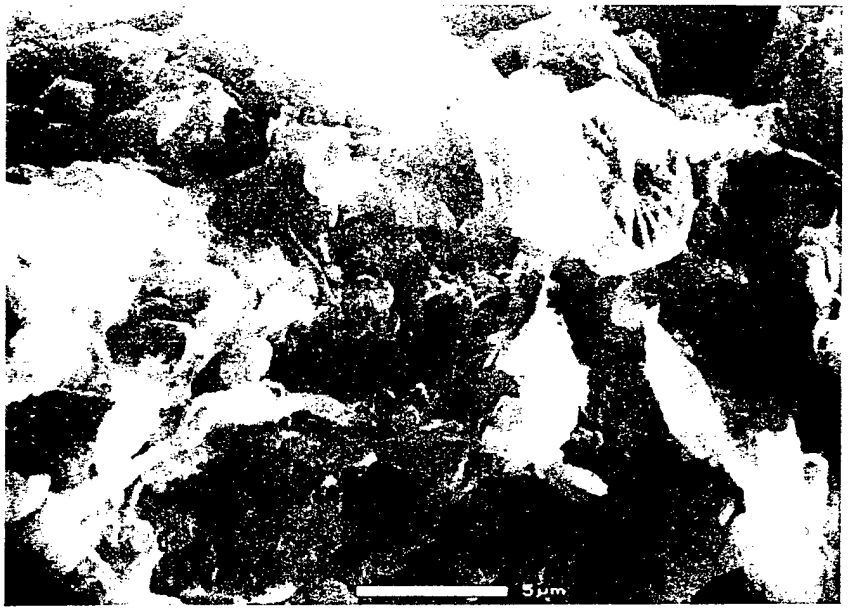

Plate 14:10.11 A natural biofilm on Nylon(TM) rope: heavy biopolymer matrix, unfixed sample. Bar = 5 µm.

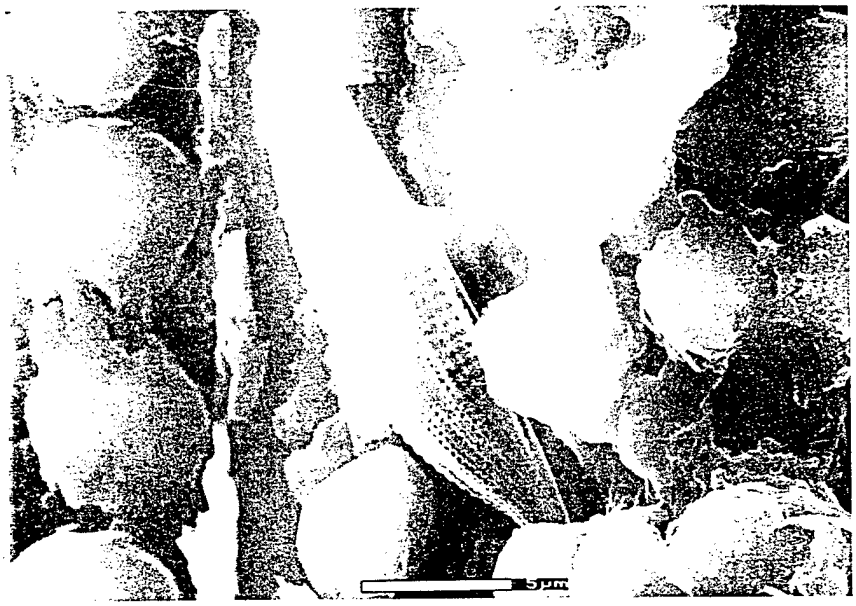

Plate 14:10.12 The surface of marsh grass, showing (from left to right) leaf nodules, a diatom and biopolymer; unfixed sample. Bar = 5 μm.

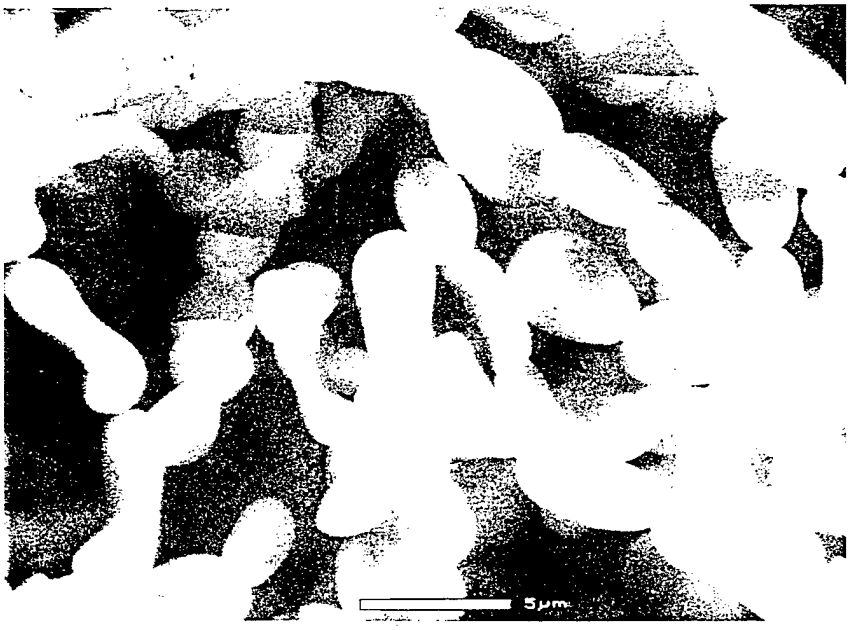

Plate 14:10.13 Fungus of the genus *Hormecium* on agar; live specimen. Note the collapse of the conidia. This specimen maintained viability (as evidenced by continued growth) after ESEM examination. Bar = 5 μm.

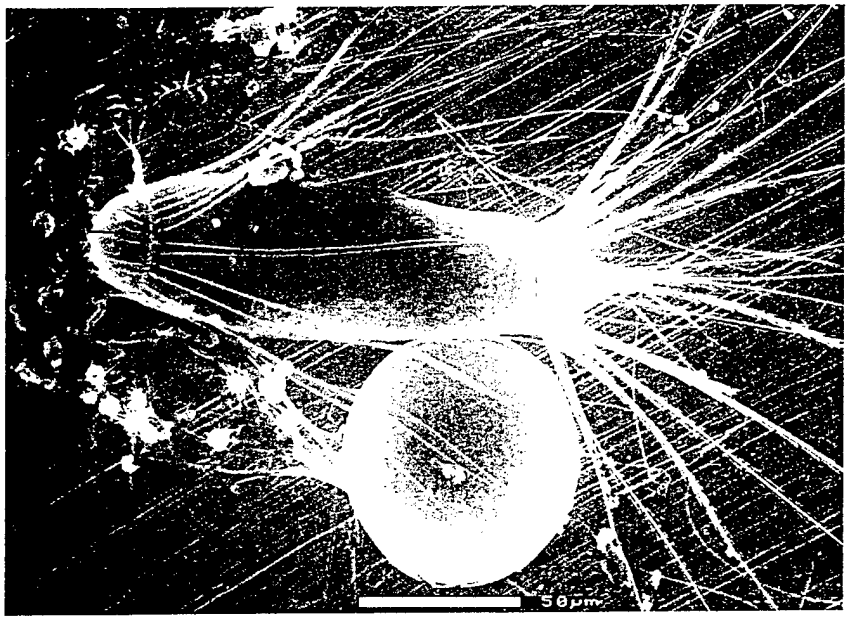

Plate 14:10.14 Marine zooplankton, fixed with buffered glutaraldehyde. Note numerous bacterial cells visible just beneath its surface. Bar = 50 µm.

Plate 14:10.15 The result of not rinsing a fixed, marine sample. Note the heavy coating of polymerized glutaraldehyde over the emerging salt crystals. Bar = 50 µm.

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